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# Transporters involved in uptake of di- and tricarboxylates in *Bacillus subtilis*

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**Abstract**

Di- and tricarboxylates found as intermediates in the tricarboxylic acid cycle can be utilized by many bacteria and serve as carbon and energy source under aerobic and anaerobic conditions. A prerequisite for metabolism is that the carboxylates are transported into the cells across the cytoplasmic membrane. *Bacillus subtilis* is able to metabolize many di- and tricarboxylates and in this overview the available data on all known and putative di- and tricarboxylate transporters in *B. subtilis* is summarized. The *B. subtilis* transporters, that are of the secondary type, are discussed in the context of the protein families to which they belong. Available data on biochemical characterization, regulation of gene expression and the physiological function is summarized. It is concluded that in *B. subtilis* multiple transporters are present for tricarboxylic acid cycle intermediates.

## Introduction

Di- and tricarboxylates found as intermediates in the tricarboxylic acid cycle (TCA cycle, citric acid cycle, or Krebs cycle) can be metabolized by many bacteria and serve as carbon and energy source. Under aerobic conditions the substrates are oxidized to $CO_2$ and $H_2O$ by running a full or partial TCA cycle during which free energy is conserved in the form of reducing equivalents that are used to generate proton motive force (pmf) and ATP in oxidative phosphorylation. Under anaerobic conditions multiple pathways exist. The $C_4$-dicarboxylic acids fumarate and L-malate, that is dehydrated to fumarate by fumarase, serve as energy source through fumarate respiration which leads to the generation of a pmf, high enough to drive the synthesis of ATP by $F_0F_1$-ATPases (for review see Janausch et al. 2002, Kroger et al. 2002). Succinate produced during fumarate respiration is exchanged for external fumarate without the cost of metabolic energy.

The tricarboxylate citrate is used under anaerobic conditions following different pathways (for enterobacteriarae review by Bott 1997). In all cases, citrate is first converted to oxaloacetate by citrate lyase. Bacteria that lack this enzyme, as is the case for *Bacillus subtilis*, do not grow anaerobically on citrate. After the conversion of citrate to oxaloacetate several pathways are possible. In *Escherichia coli*, oxaloacetate is converted to malate and, subsequently, to fumarate by malate dehydrogenase and fumarase, respectively. Fumarate is metabolized further by fumarate respiration. A co-substrate like glucose is required to supply the reducing equivalents. In *Klebsiella pneumoniae*, no co-substrate is needed. Oxaloacetate is converted to pyruvate and carbon dioxide by oxaloacetate decarboxylase. This membrane embedded enzyme functions as a $Na^+$ pump, thereby conserving the free energy released in the decarboxylation reaction in the form of a sodium ion gradient (for review see Dimroth et al. 2001). The sodium ion motive force is used to drive the uptake of citrate, or can be converted into a pmf by the action of $Na^+/H^+$ antiporters. Subsequently, pyruvate is converted to

acetyl-CoA, which is converted to acetylphosphate and, finally, to acetate, a process which yields one mol of ATP per mol of citrate (Bott 1997).

Lactic acid bacteria use fermentative pathways to metabolize the di- and tricarboxylates malate and citrate. In *Lactococcus lactis* malate is decarboxylated to yield lactate. The enzyme is cytoplasmic and, in contrast to the membrane bound $Na^+$-pump in *K. pneumoniae*, not energy conserving. The uptake of malate is coupled to the exit of its decarboxylation product lactate (precursor/product exchange) which results in the formation of a membrane potential of physiological polarity. Furthermore, during the decarboxylation step a cytoplasmic proton is consumed which results in a pH gradient over the cell membrane. Taken together the pathway, termed malolactic fermentation, results in a pmf that is large enough to drive ATP synthesis via the $F_0F_1$-ATPase (Poolman et al. 1991; Bandell et al. 1997). In a similar, but more complex manner, citrate is metabolized in *Leuconostoc mesenteroides* and *L. lactis* by citrolactic fermentation (Marty-Teysset et al. 1996). Similar as in malolactic fermentation, divalent citrate is taken up in exchange for monovalent lactate. The pathway in the cytosol converting citrate into lactate requires three different enzymes and requires co-metabolism of glucose (Marty-Teysset et al. 1995, 1996).

For all metabolic pathways described above it is essential that the di- and tricarboxylates be transported across the cytoplasmic membrane. All known and putative transporters of di- and tricarboxylates are secondary transporters; they derive energy from the chemiosmotic gradient of protons or sodium ions to accumulate the substrate against a concentration gradient. Typical for the anaerobic breakdown pathways is the coupling of the uptake of the growth substrate to the excretion of the end product of the pathway, i.e. fumarate/succinate exchange, citrate/succinate exchange, citrate/lactate exchange, and malate/lactate exchange.

Di- and tricarboxylate uptake in *B. subtilis* has been studied in the seventies and early eighties of the last century. For $C_4$-dicarboxylates two uptake systems were described. One is a L-malate uptake system that is induced by L-malate in the medium. It was reported to be responsible for growth on L-malate as sole carbon and energy source (Willecke and Lange 1974). The second system is less specific and was claimed to transport L-malate, succinate and fumarate. It was found in cells grown on yeast extract (Ghei and Kay 1973), in the presence of citrate (Ghei and Kay 1972), or expressed constitutively (Bisschop et al. 1975). The latter study in membrane vesicles showed that the transporter was of the secondary type. Similarly, it was demonstrated that a secondary transporter catalyzes the uptake of the tricarboxylate citrate in *B. subtilis* (Bergsma and Konings 1983). Remarkably, uptake of citrate was dependent on the availability of divalent metal ions (Warner et al. 2002b). These early reports offer no information on the identity of the transporters that are responsible for the uptake of the solutes. The availability of modern molecular biology techniques has greatly facilitated the identification and characterization of the proteins involved in specific metabolic pathways. Moreover, the complete genome sequence of *B. subtilis* (Kunst et al. 1997) yielded valuable information on all the transport capabilities of this bacterium (Saier et al. 2002).

In this review an overview is given of the di- and tricarboxylate transporters found in *B. subtilis* and the protein families to which these transporters belong (summarized in Table 1). Available data on functional properties, regulation of transcription and physiological function of the individual proteins are discussed.

*Table 1.* Transporter families and individual transporters of *B. subtilis* 168 discussed in this review.

| Family | TC system[a] | Transporter | Gene | GI #[b] |
|---|---|---|---|---|
| MeCit | 2.A.11. CitMHS | CitM | *yflN* | 16077828; 2443237 |
| | | CitH | *yxiQ* | 16080957 |
| | | YraO | *yraO* | 16079739;2108284 |
| DctA | 2.A.23. DAACS | DctP | *ydbH* | 16077514 |
| 2HCT | 2.A.24. CCS | MaeN | *yufR* | 16080210;7446780 |
| | | CimH | *yxkJ* | 16080928;7446781 |
| NhaC | 2.A.35. NhaC | MleN | *yqkI* | 16079413;1731094 |
| DASS | 2.A.47. DASS | YflS | *yflS* | 16077824 |

[a] Transport Commission family number and name (Saier 2000)[b] NCBI Protein database GI number (www.ncbi.nlm.nih.gov).

## MeCit family

The MeCit family currently consists of 12 known proteins that are found exclusively in bacteria. Some are found in typical pathogenic bacteria, like *Staphylococcus aureus* and *Neisseria meningitidis*, others in typical soil bacteria like *Streptomyces coelicolor* or *B. subtilis*. Two members of the family have been characterized in some detail, CitM and CitH of *B. subtilis*. The transporters transport tricarboxylates in complex with divalent cations. Both were shown to be proton-coupled symporters. The hydropathy profiles of members of the family contain 11 to 12 hydrophobic regions, suggesting the same number of putative transmembrane segments (TMSs). The membrane topology of the family has not been determined experimentally. *B. subtilis* contains in total three proteins that belong to the MeCit family, besides CitM and CitH, the protein coded by open reading frame *yraO*, which has not been characterized.

### *Characterization*

CitM and CitH, designated YflN and YxiQ in the Subtilist database, respectively, and YraO of *B. subtilis* share 52 to 61% identical residues and, additionally, 18 to 25% similar residues. CitM and CitH have been successfully cloned and functionally expressed in *E. coli* (Boorsma et al. 1996; Krom et al. 2000). However, functional expression of *yraO* in *E. coli* has failed so far (B.P. Krom, unpubl.). Using right-side-out (RSO) membrane vesicles, CitM and CitH were shown to be secondary transporters that are driven by the transmembrane pH gradient, $\Delta pH$, and by the membrane potential, $\Delta\Psi$, inside negative (Boorsma et al. 1996). This observation indicates that at least one net positive charge is transported into the cell.

At first, CitH was identified as a transporter for free citrate (Boorsma et al. 1996). However, a more recent study has shown that CitH is a $Ca^{2+}$-citrate transporter that also accepts $Ba^{2+}$ and $Sr^{2+}$ complexed to citrate but not free citrate (Krom et al. 2000). The apparent affinities ($K_{app}$) for the different complexes were very similar, ranging from 33 to 50 μM. In contrast, the maximal rates of transport were at least 3 fold higher for $Ca^{2+}$-citrate than for $Sr^{2+}$-citrate or $Ba^{2+}$-citrate. It was shown that $Ca^{2+}$ is co-transported into the cell.

CitM has been shown to transport $Mg^{2+}$-citrate, but also $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$ and $Zn^{2+}$-citrate. Similar as observed for CitH, the $K_{app}$'s of the cloned transporter in *E. coli* were very similar for the different complexes ranging from 35 to 63 μM and are in line with the $K_{app}$ of 40 μM measured in *B. subtilis* vesicles for Mg-citrate (Bergsma and Konings 1983). The maximal rate of transport ranged from 214 to 661 pmol/mg protein · min for $Mn^{2+}$-citrate and $Co^{2+}$-citrate, respectively (Krom et al. 2000). It has been shown that the divalent cation is transported into the cell, i.e. the complex of metal and citrate is the transported species. Besides complexes of citrate with divalent metal ions, CitM also transports isocitrate in complex with divalent metal ions (Warner et al. 2002b).

Summarizing, both CitM and CitH transport a metal-citrate complex, but with complementary metal specificity. It is possible that the two transporters distinguish metal ion-citrate complexes based on the ionic radii of the metal ions. CitH would transport bulky ions with ionic radii greater than 0.98 Å, while CitM would transport the smaller ions (Krom et al. 2000). Since transport by both proteins was shown to be electrogenic and the Me-citrate complex is monovalent anionic, at least two protons are co-transported with the Me-citrate complex.

### *Regulation of expression*

Expression of the *citH* gene was studied using the LacZ-fusion approach by which the promoter region of *citH* was fused upstream of the gene for β-galactosidase (*lacZ*). Growth of the recombinant strain on plates in the presence of many different di- and tricarboxylates revealed a low level of expression, but no clear induction pattern. Similarly, a low level of expression was observed in exponentially growing cells which correlated with the low citrate transport activity in the presence of $Ca^{2+}$, $Sr^{2+}$ and $Ba^{2+}$ in the cells (Krom et al. 2000). On the other hand, a DNA array study using 24 two-component regulatory systems present on the genome of *B. subtilis* indicated that the YufLM sensor kinase-response regulator pair would be involved in CitH induction (Kobayashi et al. 2001). Interestingly, many genes induced via YufLM are members of the ComK regulon involved in competence (Kobayashi et al. 2001). Possibly CitH expression is induced during the development of competence.

The regulation of expression of *citM* has been studied extensively. *CitM* expression requires the presence of $Mg^{2+}$ and citrate or isocitrate in the

medium (Warner et al. 2000; Yamamoto et al. 2000). $Mn^{2+}$-, $Ni^{2+}$-, $Zn^{2+}$- and $Co^{2+}$-citrate were also capable of induction, albeit to lower levels. The induction is mediated by a classical two-component signal transduction system, consisting of a sensor, CitS encoded by *yflR*, and a response regulator, CitT encoded by *yflQ*, both of which are located upstream of *citM* (Figure 1). CitT is the DNA binding transcriptional regulator and has been shown to bind to two regions in the promoter region of *citM* (Yamamoto et al. 2000). The induction by citrate is overruled by the presence of carbon sources that are more easy to metabolize, like glucose, glycerol and inositol (Warner et al. 2000). Also the combination of succinate and glutamate and some component(s) in Luria Bertani broth were shown to repress CitM expression (Warner et al. 2002a, 2003). Repression is mediated by the classical Carbon Catabolite Repression system (CCR) as has been shown for numerous other proteins in *B. subtilis* (Hueck and Hillen 1995). A functional Catabolite Responsive Element (CRE) is located within the promoter region of CitM (Miwa et al. 2000; Warner et al. 2000; Yamamoto et al. 2000).

The third member of the MeCit family in *B. subtilis*, encoded by *yraO* gene, could not be shown to be expressed under any of many conditions tested using the LacZ promoter fusion approach (B.P. Krom, unpubl.). Either this gene is expressed under very special conditions, or the gene is cryptic.

*Physiological function*

*B. subtilis* grown in continuous cultures with glucose as sole carbon source results in acid production caused by overflow of the TCA-cycle, and consequently, rapid acidification of the medium which inhibits growth (Goel et al. 1995). Co-metabolism of glucose and citrate resulted in lower acid production and a higher growth yield due to a better coordination between glycolysis and the TCA cycle. It has been proposed that elevated levels of $Ca^{2+}$ in the cell might result in inhibition of pyruvate kinase activity, thus slowing down glycolysis. A specific $Ca^{2+}$-citrate transporter was thought to be responsible for this elevated level of $Ca^{2+}$ in the cell. If this were indeed the case, CitH may very well be this $Ca^{2+}$-citrate uptake system.

CitM is the transporter involved in the growth of *B. subtilis* on citrate (Yamamoto et al. 2000) and isocitrate (Warner et al. 2002b) as sole carbon source. A CitM deletion strain lost the ability to grow on citrate and isocitrate and both carbon sources induced expression of the transporter when present in the medium. Catabolite repression by other medium components exerted on CitM expression further confirm the role of CitM during growth on citrate and isocitrate. The effect of CitM expression in the presence of citrate complexed to toxic heavy metals like $Ni^{2+}$, $Zn^{2+}$ and $Co^{2+}$, on the growth of *B. subtilis* dramatically increased the toxic effects of these metals (Krom et al. 2002). The fact that CitM expression is induced best by the $Mg^{2+}$-citrate complex as compared to the $Ni^{2+}$, $Zn^{2+}$ and $Co^{2+}$-citrate complexes, together with the toxic effects in the presence of these heavy metal-citrate complexes, suggest that $Mg^{2+}$-citrate is the natural substrate for CitM.

Bergsma and Konings studied $Me^{2+}$-dependent citrate uptake in membrane vesicles prepared form *B. subtilis* W23 cells grown in the presence of citrate (Bergsma and Konings 1983). At that time it was not recognized that citrate transport in *B. subtilis* could be mediated by at least three different transporter proteins and the observed metal dependence is best explained by similar levels of expression of both CitM and CitH. The uptake of citrate was high in the presence of both $Mg^{2+}$ and $Ca^{2+}$. In *B. subtilis* strain 168, the contribution of the $Ca^{2+}$-dependent uptake was much smaller (Krom et al. 2000), suggesting a different regulation of expression in the W23 and 168 strains.

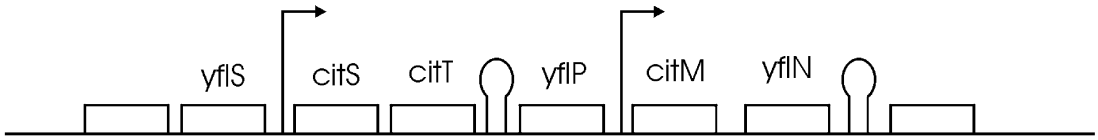


*Figure 1.* Schematic overview of the region on the *B. subtilis* genome that contains the *citM* gene. Arrows indicate confirmed or putative promoter regions, while terminators of transcription are indicated by loop structures. *CitST* code for the sensor kinase and response regulator respectively, *citM* codes for the $Mg^{2+}$-dependent citrate transporter.

## DctA family

In bacteria, four families of secondary transporters specific for $C_4$-dicarboxylates can be distinguished; the DctA family, members of which are expressed under aerobic conditions, and the three Dcu families, DcuA, DcuB and DcuC, members of which are expressed under anoxic or anaerobic conditions. No homologues of either one of the Dcu families were identified in the genome of *B. subtilis*, while one homologue of the DctA family was found, and, unfortunately, designated DctP (Asai et al. 2000). The DctA family is a subfamily of the large dicarboxylate/aminoacid:cation symporter (DAACS) family of transporters found in bacteria, archaea and eukaryotes (Saier 2000; Janausch et al. 2002). The bacterial DctA subfamily homologues share at least 40% identical residues (Slotboom et al. 1999a) and are found in Gram-positive and Gram-negative aerobic or facultative anaerobic bacteria. All members use either $H^+$ or $Na^+$ as coupling ion and are around 450 amino acids in length (for a recent review see Janaush et al. 2002). *Rhizobium meliloti* DctA is reported to contain 12 TMS based on a series of LacZ fusions (Jording and Puhler 1993). Members of the DctA subfamily share between 27% and 40% amino acid sequence identity with members of the glutamate transporter subfamily of the DAACS family. The latter proteins contain 8 TMSs and two reentrant loop-like structures that resemble the pore-loop structures found in ion channels (Grunewald et al. 1998; Slotboom et al. 1999b, 2001). Because of significant evolutionary relationship between the two subfamilies, a similar membrane topology most likely applies to members of the DctA subfamily.

### *Characterization*

To date no cloning and functional characterization of DctP of *B. subtilis* has been reported. Growth studies (see below) suggest that DctP transports fumarate and succinate and, possibly, L-malate. A study by Bisschop *et al.* using membrane vesicles prepared from cells grown under conditions that later were shown to result in expression of DctP revealed apparent affinities for fumarate, succinate and L-malate of 7.5 μM, 4.3 μM, and 13.5 μM, respectively (Bisschop et al. 1975). Competitive inhibition of uptake by the three substrates suggested that they were all transported by one and the same transporter. It cannot be excluded that transporters other than DctP might have been involved in the uptake of L-malate as well. Transport of the $C_4$-dicarboxylates in the membrane vesicles was sensitive to uncouplers suggesting pmf driven transport.

### *Regulation of expression*

The *dctP* gene is transcribed both in a single mRNA and polycistronically together with two genes coding for the two-component signal transduction system DctSR (Figure 2) (Asai et al. 2000). The two-component system DctSR (the *ydbFG* genes) is required for induction of DctP together with the product of the *dctB* gene that is located immediately upstream of the *dctSRP* operon. DctB is homologous to the periplasmic binding protein of the binding protein dependent secondary transporter family (or TRAP family (for review see Kelly and Thomas 2001)), but is not needed for transport catalyzed by DctP. It was postulated that DctB might bind the substrate with high affinity and, subsequently, would activate the DctSR two-component system (Rabus et al. 1999; Asai et al. 2000).

Expression of *dctP* can be found in minimal medium in the presence of a small amount of yeast extract. Interestingly induction is prevented by the addition of mM concentrations of L-malate in the growth medium (Asai et al. 2000) while this may be one of the substrates of the transporter. It is remark-

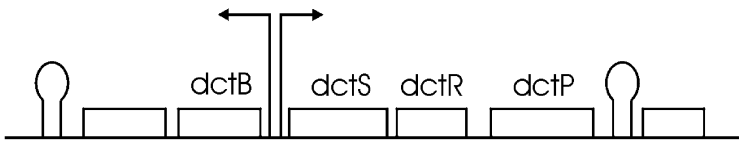


*Figure 2.* Schematic overview of the region on the *B. subtilis* genome that contains the *dct* operon. Arrows indicate the direction of transcription, terminators of transcription are indicated by loop structures. *DctB* codes for the periplasmic binding protein, *dctSR* for the sensor kinase and response regulator, respectively, and *dctP* codes for the transporter

able to see that the substrates succinate and fumarate do not induce transcription of the transporter above levels found when *B. subtilis* was grown on minimal medium with a small amount of yeast extract. Perhaps the amount of inducer in yeast extract is high enough to induce gene expression.

A functional CRE sequence located in the promoter region of *dctP* has been identified (Miwa et al. 2000) indicating that expression is under control of Carbon Catabolite Repression. In an older study it was demonstrated that malate uptake activity was repressed by the presence of glucose in the medium (Ghei and Kay 1973).

### *Physiological function*

DctP is required for growth of *B. subtilis* on fumarate and succinate, but not on L-malate. A possible explanation for this phenotype might be that multiple systems are capable of L-malate uptake while probably only one system is responsible for the uptake of succinate and fumarate. The auxotrophic phenotypes for succinate and fumarate, *suc*$^-$ and *fum*$^-$, respectively, were obtained in strains in which the two-component system was deleted (*dctS*$^-$, *dctR*$^-$) as well as in the strain in which the binding protein was deleted (*dctB*$^-$). DctP is likely to be the $C_4$-dicarboxylate transporter that is expressed in cells grown on yeast extract (Ghei and Kay 1972, 1973; Willecke and Lange 1974; Bisschop et al. 1975). Interestingly, in one of these studies a dicarboxylate binding protein has been identified, purified and characterized (Kay 1981). The binding protein had an apparent molecular mass of 41 kDa and could be extracted from membrane vesicles with detergents indicating the presence of a membrane anchor. The purified binding protein was shown to bind L-malate. The predicted molecular mass of DctB is 40 kDa. It is quite possible that DctB is the $C_4$-dicarboxylate binding protein described by Kay (Kay 1981).

## 2HCT family

The 2-hydroxycarboxylate transporter (2HCT) family currently consists of 17 members that are solely found in bacteria. Some members of the 2HCT family have been studied extensively. The family contains $Na^+$- and $H^+$-symporters (CitS of *K. pneumoniae*, MaeN and CimH of *B. subtilis* and MaeP of *Streptococcus bovis* (van der Rest et al. 1992; Kawai et al. 1997; Wei et al. 2000)), but also citrate/lactate and malate/lactate exchangers found in lactic acid bacteria (CitP of *Lc. mesenteroides* and MleP of *L. lactis* (Marty-Teysset et al. 1995; Bandell et al. 1997)). The latter transporters are involved in secondary metabolic energy generation under physiological conditions (citrolactic and malolactic fermentation, respectively (Poolman et al. 1991; Lolkema et al. 1996; Marty-Teysset et al. 1996)). Recently, a second member of the family was described in *K. pneumoniae*, termed CitW, that catalyzes citrate/acetate exchange (Kästner et al. 2002). Substrate specificity studies revealed that the symporters in the family are very specific, transporting only citrate or L-malate, while the exchangers catalyze transport of a wide range of 2-hydroxycarboxylates (Bandell et al. 1997).

The hydropathy profile of the amino acid sequence of the members of the family shows 12 hydrophobic regions long enough to traverse the membrane. Studies of CitS of *K. pneumoniae* revealed that 11 of these span the membrane (van Geest et al. 1999; van Geest and Lolkema 2000). The hydrophobic region between TMSs V and VI is located externally. Studies on the exchangers CitP of *L. mesenteroides* and MleP of *L. lactis* revealed that the last cytoplasmic loop and the C-terminal outgoing TMS XI are involved in substrate specificity (Bandell and Lolkema 2000b). A conserved arginine in TMS XI was shown to directly interact with one of the carboxylates of the substrate (Bandell and Lolkema 2000a, Krom et al. 2003). Conserved residues in the cytoplasmic loop preceding TMS XI were shown to be accessible from the outside of the cell. It is proposed that this cytoplasmic loop is part of the structure that forms the translocation pathway through the protein.

### *Characterization*

Two members of the 2HCT family, MaeN and CimH, are found in *B. subtilis* and have been functionally characterized. MaeN, designated YufR in the Subtilist database, shows the highest sequence identity to MaeP of *S. bovis*, a $H^+$ dependent L-malate transporter (Kawai et al. 1997). *MaeN* was cloned and functionally expressed in *E. coli* (Wei et al. 2000) and shown to code for a $Na^+$ coupled L-malate transporter. *E. coli* cells deficient in malate transport could be complemented for transport of L-malate by MaeN expression. The cells were unable to grow on minimal medium containing fumarate, succinate or citrate,

indicating that these di- and tricarboxylates are not substrates of MaeN (Wei et al. 2000).

CimH, designated YxkJ in the Subtilist database, has been cloned and functionally expressed in *E. coli* (Krom et al. 2001). *E. coli* cells deficient either in malate or citrate transport could be complemented by expression of CimH, indicating that both citrate and L-malate are substrates of this transporter. CimH is capable of citrate and L-malate symport in membrane vesicles, while L-citramalate binds to the protein but is not transported. Kinetic analysis revealed a high affinity for citrate, but a low maximal rate, while the affinity for L-malate was low, but the maximal rate high. Therefore, CimH is a high affinity, low capacity citrate transporter and a low affinity, high capacity L-malate transporter. Citrate is transported in the free anionic state, in contrast to two other citrate transporters of *B. subtilis* (see MeCit family) that require divalent cations for transport. Like most 2-HCT members, CimH is highly stereoselective, recognizing the S-, but not the R-enantiomers of malate and citramalate. CimH catalyzes electroneutral $H^+$-symport; transport is driven by a pH gradient $\Delta$pH and not by the membrane potential $\Delta\Psi$. Since at pH 6, the condition of the experiments, divalent citrate and malate are the most prevalent anionic species, most likely two protons are co-transported.

*Regulation of expression*

Expression of *maeN* is induced by L-malate in the medium (Asai et al. 2000). The *yufLM* genes that code for a two component sensor system and that are located upstream of the *maeN* gene would be a potential candidate for the sensor system (Figure 3). Based on amino acid sequence identity the sensor, YufL, is clustered in the CitA family of sensors for $C_4$-dicarboxylates (Janausch et al. 2002) together with the $C_4$-carboxylate sensors DcuS of *E. coli* (35% identical residues), and citrate sensors CitS (31% identity) and CitA (25% identity) of *B. subtilis* and *K. pneumoniae*, respectively (Kaspar et al. 1999; Yamamoto et al. 2000). Nevertheless, a DNA array study did not identify *maeN* as a potential target for YufL regulation. In fact, none of 24 two component regulatory systems analyzed could be linked to induction of MaeN expression (Kobayashi et al. 2001). The uptake of malate is repressed by the presence of glucose in the medium (Ghei and Kay 1973). Although not included in an evaluation of 126 putative CRE-sequences in *B. subtilis* (Miwa et al. 2000) a putative CRE-sequence is present in front of *maeN* with only two mismatches (Figure 4) with the consensus CRE-sequence (Weickert and Chambliss 1990).

A *B. subtilis* strain containing a fusion of the promoter region of *cimH* and the gene coding for β-galactosidase (LacZ fusion) did not show induction of expression on solid nor in liquid medium supplemented with a variety of di- or tricarboxylates, including citrate and L-malate. Cells harvested in the exponential growth phase grown on a minimal medium (Krom et al. 2000) showed a low citrate uptake activity in the presence of the chelator EDTA, suggesting a low, constitutive level of expression. The promoter of *cimH* contains a functional CRE sequence suggesting that expression is subject to Carbon Catabolite Repression (Miwa et al. 2000). Interestingly, at the position of the ribosome binding site (RBS) in front of *cimH* of the 2HCT family and *citH* of the MeCit family, a completely conserved stretch of ten nucleotides are present at exactly the same distance from the translation start site (Figure 4). Only two other genes on the *B. subtilis* genome share this sequence at the same position; *dctB*, the $C_4$-dicarboxylate periplasmic binding protein involved in induction of *dctP* (see DctA subfamily), and *mleA*, encoding the malolactic enzyme found in the *mleAN* operon (see NhaC family below). Possibly, this sequence constitutes a rather long conserved RBS. Alternatively it might represent some sort of regulatory region. The relevance of this observation is not clear.

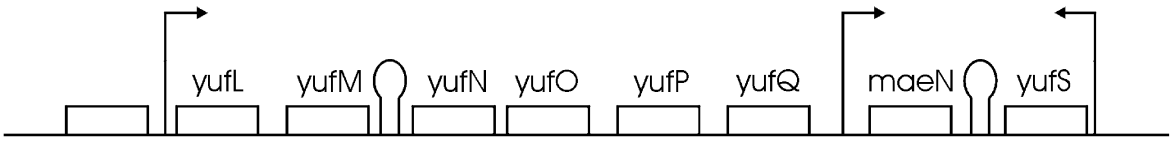


*Figure 3.* Schematic overview of the region on the *B. subtilis* genome that contains the *maeN* gene. Arrows indicate the direction of transcription, putative terminators of transcription are indicated by loop structures. *YufLM* encode the sensor kinase and response regulator, respectively, *maeN* encodes the malate transporter.

**A**

```
CRE         TGWAANCGNTNWCA
            ||| |||||||||
maeN        TGTTAACGCTTTCT
```

**B**

```
cimH        CTTAACATAGTGAACAAT-TTCACTGCATCAAG--AATAGGGGAGG-TTTTTCAATG
citH        ---AAGGATGGAAGCGCTTACCAAAAAAGCTGTTGAATAGGGGAGG-TCATATCATG
dctB        TTTACGAATGTCATAGCC-CCCATTATCCTTAAC--ATAGGGGAGG-GCTTTTCATG
mleA        ---AACAATACAGTTAAA-GCCGAAAAACTAGGCTAATAGGGGAGGATCACTGAATG
               *           *           **********        ***
```

*Figure 4.* Panel A. Alignment of consensus CRE sequence upstream of the *amyE* gene and the putative CRE sequence found in the *maeN* promoter region. N and W represent any base and A or T, respectively. Vertical lines indicate identical bases. Panel B. Alignment of the 50 base pairs preceding the ATG start codon (underlined) of four genes involved in carboxylate transport and metabolism in *B. subtilis*. *CimH* codes for a citrate/malate transporter, *citH* for a $Ca^{2+}$-citrate transporter, *dctB* for a dicarboxylate binding protein, and *mleA* for a malolactic enzyme. The alignment was prepared using the Clustal X program (Jeanmougin et al. 1998). Conserved nucleotides are marked with an asterix.

*Physiological function*

The 2HCT family contains proteins that function as symporters or precursor/product exchangers. The latter are involved in the generation of metabolic energy in lactic acid bacteria, (Poolman et al. 1991; Lolkema et al. 1996; Marty-Teysset et al. 1996). MaeN and CimH probably function as L-malate and citrate/L-malate symporters, respectively, under physiological conditions (Wei et al. 2000; Krom et al. 2001). The observation that CimH is a high affinity, low capacity citrate transporter and a low affinity, high capacity L-malate transporter could mean that the physiological function of CimH is two-fold. Firstly, CimH might function in the uptake of citrate under conditions where citrate in the medium is present in its uncomplexed state. Secondly, CimH might function as an L-malate transporter at high concentrations of L-malate, while another transporter, possibly DctP, takes over at lower concentrations. Microorganisms are known to use both high and low affinity transporters for the same substrate that are differentially expressed dependent on the external concentration of the substrate (e.g. the iron transporters of *Candida albicans* (Ramanan and Wang 2000)).

## NhaC family

$Na^+/H^+$ antiporters are very abundant in nature and believed to play a role in pH and $Na^+$ homeostasis (Padan and Schuldiner 1994; Padan et al. 2001). The proteins are distributed over a number of different gene families, but in *B. subtilis* only two members of the NhaC family are present (Saier et al. 2002). The NhaC family of $Na^+/H^+$ antiporters is relatively small, with members found in bacteria and in archaea. The proteins are around 460 amino acids long and predicted to contain 12 putative TMSs. The two members in *B. subtilis*, YheL and MleN, are the only members that have been characterized to some extent (Ito et al. 1997; Wei et al. 2000). YheL functions as a genuine $Na^+/H^+$ antiporter (not further discussed here), and MleN couples $Na^+/H^+$ exchange to the exchange of malate and lactate (Wei et al. 2000).

*Characterization*

MleN, designated YqkI in the Subtilist database, was cloned and functionally expressed in *E. coli*. Everted membrane vesicles expressing MleN took up lactate in symport with $Na^+$ ions, but only in the presence of intravesicular L-malate. Since the exchange between lactate and L-malate was electroneutral, it was concluded that MleN catalyzes the exchange of divalent L-malate and two $H^+$ for monovalent lactate and one $Na^+$ ion (Wei et al. 2000).

*Regulation of expression*

The *mleN* gene is found together with the *mleA* gene in an operon structure (Figure 5). MleA codes for malolactic enzyme that converts malate in a single step into lactate and $CO_2$. No information is available

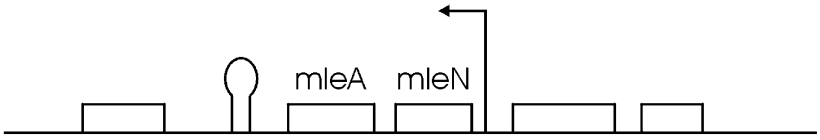


*Figure 5.* Schematic representation of the DNA region that contains the *mle* operon. The arrow indicates the direction of transcription. A putative terminator of transcription is indicated with a loop structure. *MleN* encodes the transporter, *mleA* encodes malolactic enzyme.

on the regulation of transcription of the operon. Mostly based on the proposed physiological function (see below), it was suggested that *mleN* expression would be induced under low pmf conditions. The *mleA* gene is one of the four genes on the *B. subtilis* genome that is preceded by the conserved putative RBS depicted in Figure 4.

*Physiological function*

The organization of MleN together with malolactic enzyme MleA in an operon structure suggests a similar pathway for the breakdown of malate as observed in lactic acid bacteria. Malate is taken up, decarboxylated yielding lactate and carbon dioxide, and lactate leaves the cell in exchange for malate. In lactic acid bacteria, malolactic fermentation functions in proton motive force generation (Poolman et al. 1991) or resistance against lactate under acidic conditions (Magni et al. 1999). It was proposed that in *B. subtilis* the pathway might function in $Na^+$ resistance under conditions of low pmf by the following mechanism. $H_2$-malate would enter the cell via malate-lactate exchange catalyzed by MleN and be converted into lactate and $CO_2$ by MleA. $Lactate^-$ would leave the cell in symport with $Na^+$ and in exchange for $H_2$-malate in an electroneutral transport step. Apart from the chemical conversion, the net result would be the exchange of one cytoplasmic $Na^+$ ion for one $H^+$, i.e. the decarboxylation reaction catalyzed by MleA would drive the extrusion of $Na^+$ catalyzed by MleN. It is proposed that *B. subtilis* uses this pathway as an alternative $Na^+/H^+$ antiport mechanism in $Na^+$ resistance under conditions of low pmf (Wei et al. 2000). *B. subtilis* grown on malate under conditions of low pmf requires MleN activity for growth.

## Miscellaneous transporters

The genome of *B. subtilis* (Kunst et al. 1997) contains many proteins with unknown functions. Based on homology to proteins with known function, putative transporters of di- and tricarboxylates may be assigned. One additional ORF, *yflS*, was identified that could potentially code for a transporter involved in growth of *B. subtilis* on di- and tricarboxylates. Two other ORF's coding for YoaB and CsbX are annotated in the Subtilist database as putative α-ketoglutarate transporters, but are probably not.

On the genome of *B. subtilis, yflS* is located close to the gene that codes for the $Mg^{2+}$-citrate transporter CitM (see Figure 1). The product of the *yflS* gene belongs to the ubiquitous family of divalent anion:$Na^+$ symporters (DASS (Saier et al. 2002)). All members are around 450 amino acids in size and contain 12 hydrophobic regions indicative of 12 putative TMSs (Pos et al. 1998). Members of the family transport inorganic anions, like sulfate and phosphate, but also organic anions like di- and tricarboxylates and acidic amino acids. The product of *yflS* is homologous to CitT, the citrate-succinate antiporter of *E. coli* that is expressed under anaerobic conditions (Pos et al. 1998). CitT is located in an operon involved in the metabolism of citrate under anaerobic conditions, similar to the operon for anaerobic citrate metabolism in *K. pneumoniae* (Bott 1997). These operons contain the genes coding for citrate lyase that converts citrate into oxaloacetate, an essential step in anaerobic citrate metabolism; there genes are not present on the *B. subtilis* genome. Therefore, *B. subtilis* is unlikely to be capable of anaerobic growth on citrate and YflS to be a citrate-succinate antiporter as is CitT of *E. coli*. YflS has the highest identity with SOT1 found in chloroplasts of *Spinacia oleraceae* (35% identity to CitT, 53% to SOT1). SOT1 has been extensively characterized (Weber et al. 1995) and was shown to import α-ketoglutarate in exchange for stromal malate. Besides malate also succinate, fumarate and α-ketoglutarate could be used as counter ions. Possibly YflS transports one of these substrates.

The two homologous gene products YoaB and CsbX have been annotated in the Subtilist database (http://genolist.pasteur.fr/SubtiList/) to transport α-ketoglutarate. A BLAST search (Altschul et al. 1997) reveals the highest homology to the ribitol transporter, RbtT of *E. coli* and *K. pneumoniae* and the arabinitol transporter DalT of *K. pneumoniae* and no significant

homology to known α-ketoglutarate transporters like KgtP of *E. coli*. Since no experimental data are available to support the annotation, we tentatively conclude that both CsbX and YoaB are not involved in transport of di- or tricarboxylates in *B. subtilis*.

## Conclusion

Transport of TCA cycle intermediates in *B. subtilis* is catalyzed by both proton and sodium dependent secondary transporters. A variety of protein families are involved and overlapping substrate specificities between several transporters has been found (Table 2). Multiple transport systems could be involved in growth on a certain substrate as carbon and energy source.

At least three and possibly five transporters (Table 2) transport citrate. Because *i.* CitM expression is induced by citrate in the medium, *ii.* induction is repressed via Carbon Catabolite Repression, and *iii.* the CitM deletion strain shows a *cit*$^-$ phenotype, CitM is the transporter responsible for growth on citrate as sole carbon and energy source under standard conditions. The exact roles of CitH and CimH remain unclear, however both proteins are functional citrate transporters that, under standard growth conditions, are expressed at very low levels. Until the inducing conditions for both transporters are found, their exact role in *B. subtilis* remains unclear. YraO, the third member of the MeCit family in *B. subtilis* is likely to be a citrate transporter as well, but expression of the ORF was not observed, nor has it been possible to heterologously express the gene coding for YraO. YflS is a homologue of CitT of *E. coli*, a citrate/succinate antiporter that is expressed under anaerobic conditions. Though the *B. subtilis* genome does not contain the known genes coding for citrate lyase, necessary for anaerobic citrate degradation, anaerobic growth of *B. subtilis* has recently been reported (for review see Nakano and Zuber (1998)). Possibly, YflS plays a role under these growth conditions.

*Table 2.* Transporters involved in transport of TCA cycle intermediates in *B. subtilis* 168.

| Substrate | Confirmed transporter | Putative transporter |
|---|---|---|
| citrate | CitM, CitH and CimH | YraO and YflS |
| cis-aconitate | – | – |
| isocitrate | CitM | CitH and YraO |
| α-ketoglutarate | – | – |
| succinate | DctP | YflS |
| fumarate | DctP | YflS |
| malate | MaeN, MleN, DctP and CimH | YflS |
| oxaloacetate | – | – |

Isocitrate is transported by CitM in the MeCit family. A CitM deletion strain of *B. subtilis* could not utilize isocitrate as carbon source (Warner et al. 2002b). Therefore, CitM is the transporter responsible for growth on isocitrate in *B. subtilis*. Isocitrate transport by CitM is probably dependent on the presence of the same divalent metal ions as the transport of citrate (Warner et al. 2002b). Since CitH and YraO share a high sequence identity to CitM it is possible that both proteins are also capable of isocitrate transport. However, unlike CitM, CitH was not induced by isocitrate in the medium (B.P. Krom, unpubl.).

Succinate and fumarate are both transported by DctP. A DctP knock-out strain has a *suc*$^-$ and a *fum*$^-$ phenotype indicating that DctP is the transporter involved in growth of *B. subtilis* on succinate and fumarate. Remarkably, neither succinate nor fumarate seems to be involved in the induction of DctP and the inducer that activates the two-component system DctSR remains to be identified.

The involvement of the different transporters in the metabolism of L-malate in *B. subtilis* is not exactly clear. At least four but possibly five different transport proteins are capable of L-malate transport. DctP might be involved in growth on L-malate based on studies in *B. subtilis* membrane vesicles (Bisschop et al. 1975), but since a DctP deletion strain does not show a *mal*$^-$ phenotype (Asai et al. 2000), DctP is clearly not the only transporter involved in growth on L-malate. Repression of DctP expression by millimolar concentrations of L-malate in the medium suggests that DctP may be involved at low L-malate concentrations. The low $K_m$ for L-malate transport reported by Bisschop et al. (1975) and the dependence of the sensing system that regulates expression of the gene on a binding protein (DctB) would support such a function. In contrast, expression of the $Na^+$-malate symporter MaeN in the 2HCT family is induced by L-malate in the medium and, therefore, could take over uptake of L-malate at higher concentration. Uptake of L-malate has been shown to be subject to carbon catabolite repression and the promoter region of both the *dctP* and *maeN* genes contain CRE-sequences. CimH in the 2HCT family is not induced by L-malate in the medium. The kinetic parameters, high affinity constant and maximal rate, suggest that CimH functions at high external L-malate concentrations. MleN also transports L-malate, but does so under conditions of low pmf in an exchange reaction with

Na-lactate. MleN is partly responsible for growth on L-malate under these specific circumstances, but its main function is thought to be in $Na^+$ resistance under conditions of low pmf (Wei et al. 2000). Finally, YflS is potentially involved in malate transport since it is homologous to SOT1 found in chloroplasts of *Spinacia oleraceae*, which transports, amongst others, L-malate.

In our survey of transporters of TCA cycle intermediates in *B. subtilis* we did not identify any putative or known transport systems for oxaloacetate, cis-aconitate and α-ketoglutarate. The overview provided in the paper shows clearly that *B. subtilis* is capable of using a range of di- and tricarboxylates. This can be explained by the fact that *B. subtilis* is a soil bacterium that has to be able to survive under very different growth conditions utilizing every carbon and energy source it may encounter.

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